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The S. pombe *cdc15* Gene Is a Key Element in the Reorganization of F-Actin at Mitosis

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Summary

The S. pombe *cdc15* gene is essential for cell division. *cdc15*^{ts} mutants do not form a septum, but growth and nuclear division continue, leading to formation of multinucleate cells. The earliest step in septum formation and cytokinesis, rearrangement of actin to the center of the cell, is associated with appearance of hypophosphorylated cdc15p and formation of a cdc15p ring, which colocalizes with actin. Loss of cdc15p function impairs formation of the actin ring. The abundance of *cdc15* mRNA varies through the cell division cycle, peaking in early mitosis before septation. Expression of *cdc15* in G2-arrested cells induces actin rearrangement to the center of the cell. These data implicate cdc15p as a key element in mediating the cytoskeletal rearrangements required for cytokinesis.

Introduction

Most eukaryotic cells divide symmetrically, producing daughter cells of equal size with largely similar properties. Although considerable advances have been made toward understanding the mechanisms that regulate the onset of S phase and mitosis, the control of the events that occur at the end of the cell cycle, such as the reorganization of the cytoskeleton and the initiation of cytokinesis, remains poorly understood.

Schizosaccharomyces pombe provides a simple eukaryotic model for the study of cytokinesis. Fission yeast cells grow by elongation at their ends and divide by binary fission after forming a centrally placed septum. The distribution of F-actin in S. pombe cells is intimately linked with sites of growth or cell division. During interphase, actin is located at the growing ends of the cell. At the onset of mitosis, F-actin relocates from the ends of the cell to form an equatorial ring whose position anticipates the site of septum formation (reviewed by Robinow and Hyams, 1989). After the onset of anaphase, the primary septum grows inward from the cell cortex. As septum biosynthesis proceeds, the appearance of the actin staining changes from a ring to clusters of dots. The sites of deposition of the septum material correlate well with the location of F-actin. Secondary septa are formed on either side of the primary one, which is then dissolved to effect cell separation. F-actin then relocates to the old (preexisting) end of the cell, from which growth resumes. The importance of the actin cytoskeleton is emphasized further by the finding that a strain carrying one extra copy of the actin gene shows abnormalities in cytokinesis and septum formation (Fantes, 1989).

In higher eukaryotes, a contractile ring forms around the equator of the parent cell at the end of mitosis. The sliding of actin and myosin filaments generates a force that pulls the plasma membrane inward to create the cleavage furrow, which narrows to form the midbody as division is completed (Satterwhite and Pollard, 1992; Fishkind and Wang, 1995). The telophase disc, which forms prior to the appearance of the contractile ring, has been proposed to organize contractile ring formation (Margolis and Andreassen, 1993). Proteins that are involved in the contractile ring, such as actin and myosin II, undergo dramatic rearrangments during mitosis and cytokinesis. At prophase, stress fibers break down, and the constituent actin and myosin II disperse through the cytoplasm until early anaphase, when myosin II becomes concentrated at the cortex, especially at the cell equator where the cleavage furrow will form. A number of other proteins are known to localize to the cleavage furrow, for example radixin, which is thought to provide a link between the cell cortex and the cytoskeletal elements responsible for cleavage (reviewed by Satterwhite and Pollard, 1992).

In fission yeast, as in higher eukaryotes, initiation of cytokinesis is dependent upon the onset of mitosis (Minet et al., 1979). The proteins that mediate the rearrangement of the actin cytoskeleton to form the actin ring at the onset of mitosis are likely to be among the targets for the regulatory systems that establish this dependency. Here, we show that S. pombe cells lacking cdc15p function cannot form a normal actin ring. After the onset of mitosis, cdc15p forms a ring-like structure, which colocalizes with the medial actin ring. Cdc15p is a phosphoprotein, and formation of the actin ring correlates with the appearance of hypophosphorylated cdc15p. The abundance of *cdc15* mRNA varies through the cell division cycle, peaking in early mitosis prior to septum formation, and expression of the *cdc15* gene in G2-arrested cells promotes formation of the actin ring. These data indicate that cdc15p plays a key role in regulating the initiation of cytokinesis in S. pombe.

Results

Actin Ring Formation Does Not Occur Normally in a *cdc15* Mutant

Thermosensitive mutants in the "early" septation genes, *cdc7*, *cdc11*, *cdc14*, and *cdc15*, do not form a division septum, but S phase and mitosis continue in the absence of cytokinesis (Nurse et al., 1976). To investigate whether the failure to form a division septum arises from an inability

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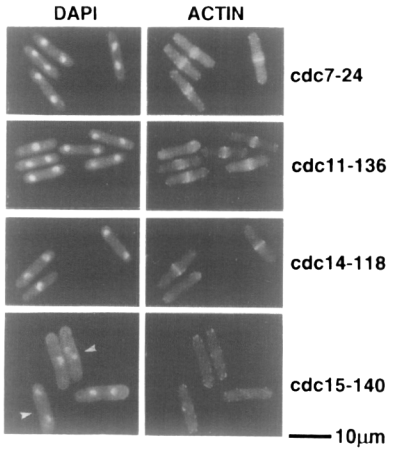

Figure 1. A Defect in *cdc15* Impairs Formation of the F-Actin Ring

cdc7-24, *cdc11-136*, *cdc14-118*, and *cdc15-140* cells were grown at 25°C to midexponential phase and then shifted to 36°C for 2 hr. Fixed cells were stained with DAPI and calcofluor (left) or with rhodamine-conjugated phalloidin (right). The arrows in *cdc15-140* indicate cells undergoing mitosis that should be forming an actin ring.

to form the actin ring, we examined actin rearrangements in these cells after shift to 36°C. *cdc7*, *cdc11*, and *cdc14* mutants all showed a normal actin rearrangement when cells entered mitosis (Figure 1), consistent with an earlier study that demonstrated that actin rings are formed at the restrictive temperature in a *cdc11* mutant (Marks et al., 1986). In contrast, in mitotic *cdc15-140* cells, F-actin was found at the tips or dispersed throughout the cell, rather than forming a ring (Figure 1). Formation of the actin ring was infrequent (approximately 5% of wild type), and the rings that were seen were faint compared with wild type.

These data show that *cdc15* activity is important for actin ring formation, while *cdc7*, *cdc11*, and *cdc14* are defective in a process subsequent to (or independent of) it. Consistent with this, analysis of double-early mutants did not reveal any genetic interactions between *cdc15* and *cdc7*, *cdc11*, or *cdc14* (Marks et al., 1992).

The *cdc15* Gene Is Essential

The *cdc15* gene was cloned by rescue of the heat-sensitive defect of a *cdc15-140* mutation at 36°C. To determine whether *cdc15* is an essential gene, we replaced part of the open reading frame by the *ura4* gene (Figure 2A). This construction was used to replace one copy of *cdc15* in a diploid. In 30 tetrads analyzed, only the two *Ura*$^-$ spores were able to form a colony, while the *Ura*$^+$ spores germinated and arrested as single cells with a phenotype similar to the *cdc15-140* mutant (data not shown), showing that *cdc15* is essential for cell division.

The *cdc15* gene encodes a 907 amino acid protein (Figure 2B). A search for known motifs identified a high scoring PEST region, which is often characteristic of proteins that are rapidly turned over (Rechsteiner, 1990), and a region at the N-terminus (amino acids 100–190) with high potential for forming coiled coils (Lupas et al., 1991), raising the possibility that cdc15p interacts with itself or other proteins. There is also an Src homology 3 (SH3) domain at

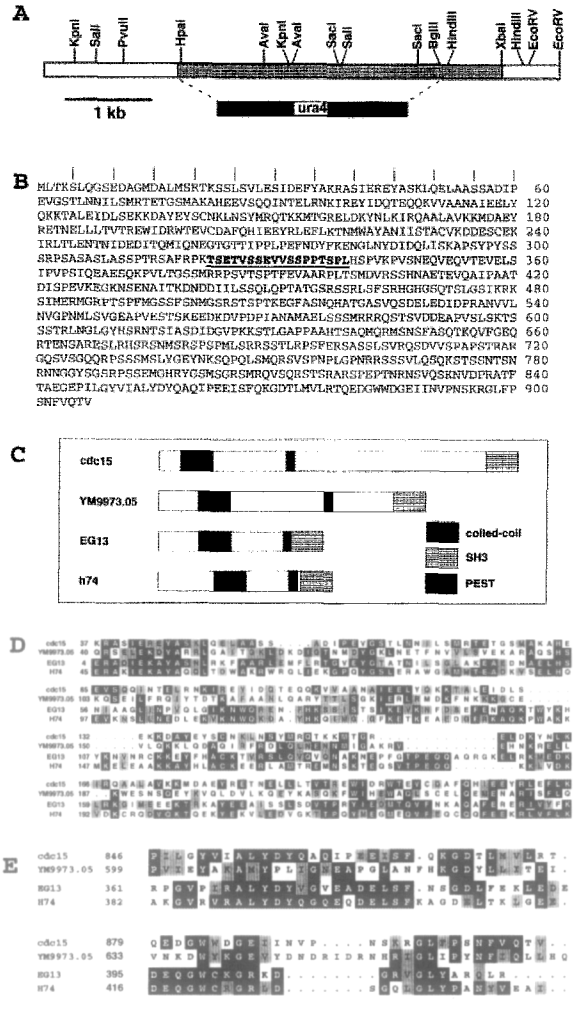

Figure 2. Restriction Map of the *cdc15* Locus and Sequence of Cdc15p

(A) Restriction map of the *cdc15* gene. The coding region is shown in gray. The region of *cdc15* replaced by *ura4* to construct the deletion is shown below.

(B) Predicted sequence of cdc15p. The one letter amino acid code is used throughout. The 907 amino acid protein has a calculated molecular weight of 99861. The highest scoring PEST region is underlined and shown in bold.

(C) Schematic comparison of proteins similar to cdc15p. The YM9973.05, EG13, and h74 proteins are shown. All contain an N-terminal domain with the potential to form coiled coils, a PEST region, and a C-terminal SH3 domain.

(D) Sequence alignment of the N-terminal helical domains of cdc15p, YM9973.05, EG13, and h74 proteins. Pairwise identities are shown in black and conservative substitutions in gray. The following substitutions were considered to be of similar character: D/E, N/Q, G/A, V/I/L/M, Y/F, K/R, S/T. The percentage identity (similarity allowing for conservative substitution) between cdc15p and YM9973.05, EG13, and h74 is 14% (25), 22% (30), and 22% (30), respectively.

(E) Sequence alignment of the SH3 domains of cdc15p, YM9973.05, EG13, and h74 proteins. Nomenclature is the same as in (D).

the C-terminus of the protein. Data base searches revealed three proteins that show similarity to cdc15p. These are an uncharacterized open reading frame from Saccharomyces cerevisiae chromosome XIII (GenBank accession number Z49213 [YM9973.05]), EG13 protein from

tapeworm (GenBank accession numbers M96564 and M96565), and h74 protein of mouse (GenBank accession number X85124). A schematic representation of the proteins is shown in Figure 2C. All have a region at the N-terminus with potential to form coiled coils and a C-terminal SH3 domain. In addition, all the proteins contain a high scoring PEST region, with the exception of h74. In the latter case, there is a weak PEST region near the C-terminus, which is indicated in Figure 2C. The greatest homology is seen in the SH3 domains and the N-terminal regions. An alignment of the N-terminal domains is shown in Figure 2D, and the SH3 domains are shown in Figure 2E. The SH3 domain of cdc15p contains all of the residues that are expected from the consensus sequence (Mussachio et al., 1994).

Cdc15p Forms a Medial Ring Coincident with Actin Ring Formation

To identify the *cdc15* protein, cdc15p, we raised an antiserum against a fusion protein between part of cdc15p and glutathione S-transferase (GST). Asynchronous wild-type cells (Figures 3A and 3C) or cells synchronized by arrest–release of a *cdc25-22* mutant (Figure 3B) were stained with antiserum to cdc15p, 4′, 6-diamidino-2-phenylindole (DAPI), to show the DNA and with rhodamine-conjugated phalloidin to reveal the location of F-actin. Normal tip, medial ring, and septum-associated actin staining was observed in interphase cells (Figure 3A, cell 1; Figure 3C), mitotic cells (Figure 3A, cells 2–4; Figure 3C), and cells undergoing cytokinesis (Figure 3A, cell 5; Figure 3B, middle and bottom panels), demonstrating that the fixation technique used gives adequate preservation of F-actin.

In mitotic cells, cdc15p staining was seen as a ring, colocalizing with the actin ring. Cells in early mitosis showed discrete cdc15p staining, but only a faint actin ring (Figure 3A, cell 2). In anaphase cells (Figure 3A, cells 3 and 4), both *cdc15* and F-actin are seen as a ring at the cell equator. The ring structure formed by cdc15p is also seen in Figure 3C, top right panel. During septum biosynthesis and cytokinesis, actin was seen as a double ring on either side of the septum, as described previously (Marks and Hyams, 1985). In contrast, cdc15p staining localized to the midzone between the daughter cells (Figure 3A, cell 5; Figure 3B, middle and bottom panels). In the population synchronized by arrest–release of *cdc25-22*, when cells are longer than wild type, double septa are observed frequently (Marks et al., 1986). In these cases, a double cdc15p ring was also seen (Figure 3B, top panels), indicating that the cdc15p ring colocalizes with the actin ring rather than the middle of the cell. Staining of *cdc15-140* after shift to the restrictive temperature showed that a cdc15p ring was not formed at mitosis (data not shown).

Interphase cells showed a diffuse cytoplasmic fluorescence with cdc15p antibody (Figure 3A, cell 1), which was apparently excluded from the nucleus. Western blotting showed that cdc15p was present at all stages of the cell cycle (see Figure 5; data not shown), eliminating the possibility that the absence of discrete cdc15p staining in interphase is due to the absence of protein. To verify the specificity of the staining, exponentially growing wild-type cells were fixed and incubated with the antibody in the presence of GST alone, the GST–cdc15p fusion protein used for immunization, or preimmune serum. The signal derived from the antiserum raised to cdc15p was competed by addition of GST–cdc15p, but not by GST alone, indicating that it is specific for cdc15p (Figure 3C). No signal was obtained with preimmune serum. Therefore, cdc15p only colocalizes with F-actin at the time of ring

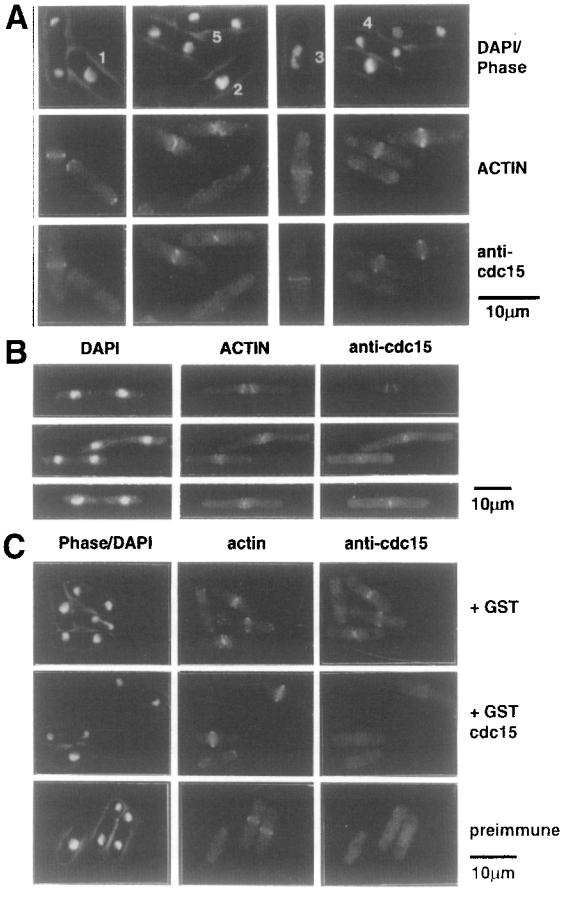

Figure 3. Immunolocalization of Cdc15p and F-Actin

(A) Localization of cdc15p and actin in asynchronous wild-type cells. Cells were grown to midexponential phase in YE medium at 25°C, fixed, and stained for cdc15p, actin, and DNA. Top panels show DAPI-stained cells, middle panels show cells stained for F-actin, and bottom panels show cells stained for cdc15p. Cell 1 is an interphase cell, cells 2–4 are mitotic cells, and cell 5 is undergoing cytokinesis.

(B) Localization of cdc15p and F-actin in synchronized *cdc25-22* cells. *cdc25-22* cells were fixed and stained as in (A) at various times after release from the G2 block. The upper panels show a cell making a double septum; the middle and lower panels show cells in the late stages of septum formation.

(C) Specificity of the immunostaining for cdc15p. Cells were grown, fixed, and stained as in (A). To assess the specificity of the affinity-purified anti-cdc15p antibody, we preincubated it with GST protein (top) or a fusion protein between a part of cdc15p and GST (middle), or cells were incubated with preimmune serum (bottom). Note that preincubation with GST alone has no effect on the signals, while preincubation with GST–cdc15p competes the signal due to cdc15p, but does not affect F-actin staining. Note also that the preimmune serum gives no specific staining.

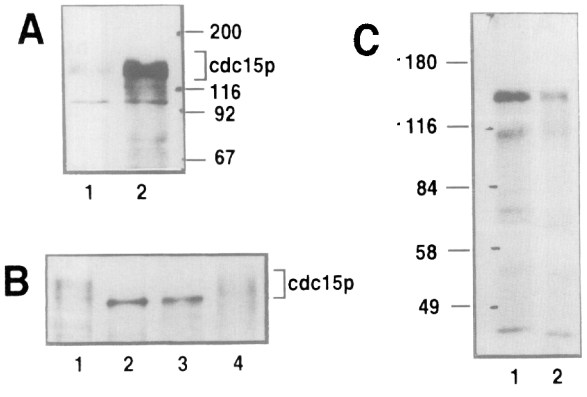

Figure 4. Cdc15p Is a Phosphoprotein

(A) Characterization of antibody to cdc15p. Protein extracts of *leu1-32* transformed with pREP3 (lane 1) or pREP3–*cdc15* (lane 2) induced for 12 hr at 29°C were Western blotted and probed with an affinity-purified anti-cdc15p antiserum. Note the increased amount of the protein bands around 130 kDa when *cdc15* is overexpressed.

(B) Phosphatase treatment converts the slower-migrating forms of cdc15p into the faster-migrating one. Synchronously dividing cells were obtained by arrest–release of *cdc25-22*. Soluble protein was extracted as described in Experimental Procedures, Western blotted, and probed as in (A). Lane 1 shows proteins in G2 just prior to the release at 36°C; lane 2 shows proteins of mitotic cells 60 min after release at 25°C; lane 3 shows a cdc15p immunoprecipitate from G2 cells that have been CIP treated; and lane 4 shows proteins from G2 cells that have been mock CIP treated. Note that when the cdc15p of G2 cells is CIP treated it comigrates with the cdc15p of mitotic cells.

(C) Cdc15p is a phosphoprotein in vivo. Synchronously dividing cells were obtained by arrest–release of *cdc25-22*. Cells (25 ml) were grown to midexponential phase in low phosphate medium and shifted to 36°C for 3 hr. [^{32}P]orthophosphate (5 mCi) was added, and the incubation was continued for 1 more hr at 36°C, after which cells were transferred to 25°C. Samples were taken at the time of shift down and 1 hr later. Proteins were extracted, and cdc15p was immunoprecipitated. After washing, the immunoprecipitates were analyzed on an SDS–polyacrylamide gel, which was fixed and dried prior to autoradiography. Note that phosphorylation of cdc15p decreases 60 min after release of the cdc block.

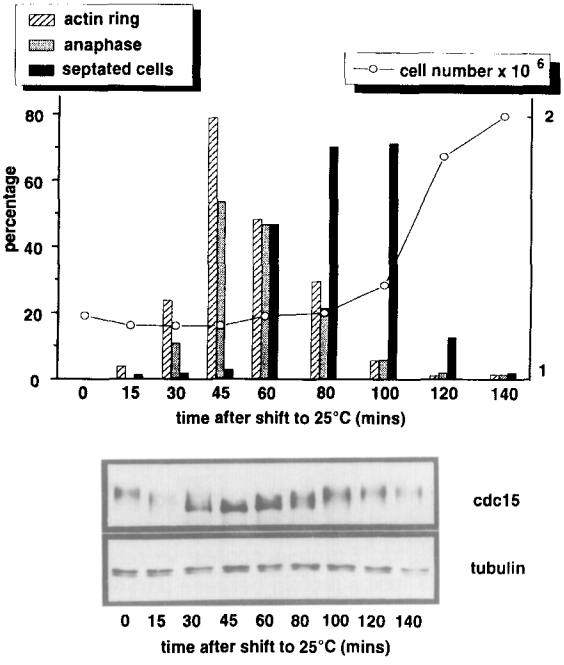

Figure 5. Formation of the Actin Ring Is Correlated with Changes in Cdc15p Phosphorylation State

Synchronously dividing cells were generated by *cdc25-22* arrest–release. Protein extracts were prepared, and cells were fixed with 4% paraformaldehyde at each timepoint. Cells were stained with rhodamine-conjugated phalloidin, DAPI, and calcofluor to determine percentage of actin rings, anaphase nuclei, and septated cells. Proteins were separated and blotted as in Figure 4A, and the blot was cut in two and probed with either affinity-purified anti-cdc15p antiserum or TAT-1 monoclonal antibody (Woods et al., 1989).

formation and septum biosynthesis, rather than during interphase, when actin is located at the growing ends of the cell. The staining pattern of cdc15p differs from those of cdc3p and cdc8p, which colocalize with actin at all stages of the cell cycle (Balasubramanian et al., 1992, 1994).

Formation of the F-Actin Ring Correlates with the Appearance of Hypophosphorylated Cdc15p

To determine whether the formation of the F-actin ring and changes in cdc15p location are associated with any modification of cdc15p, we used Western blotting to examine the protein at different stages of the cell cycle. The antiserum to cdc15p recognizes a protein of approximately 130 kDa apparent molecular mass. The amount of this protein increases in cells that express elevated levels of *cdc15*, demonstrating that it is the product of the gene (Figure 4A). Several closely spaced bands are seen in Western blots from exponentially growing cells. Immunoprecipitation of cdc15p from G2-arrested cells, followed by treatment of the precipitate with calf intestinal phosphatase, converted these to a single band, which comigrates with that seen in mitotic cells, suggesting that the slower-migrating interphase forms are generated by hyperphosphorylation of cdc15p (Figure 4B). Immunoprecipitation from ^{32}P-labeled extracts confirmed that cdc15p is a phosphoprotein (Figure 4C).

To examine whether cdc15p phosphorylation or abundance varies as cells form the division septum, we generated a synchronously dividing culture by arrest–release from a *cdc25-22* block. Cdc15p was analyzed by Western blotting, and samples were scored for formation of the actin ring, separation of nuclei, and the appearance of the division septum. In G2-arrested cells, actin was located at the tips and cdc15p was predominantly in a hyperphosphorylated form (Figure 5). 45 min after release, 80% of cells had formed an actin ring (Figure 5), whose appearance was followed closely by anaphase, but preceded formation of the division septum (80–100 min) and cytokinesis (120–140 min). Formation of the actin ring coincided with the appearance of hypophosphorylated cdc15p (30–60 min). Immunoprecipitation from ^{32}P-labeled extracts confirmed that phosphorylation of cdc15p decreased at this time (see Figure 4C). At the time of septum formation and cytokinesis (100–120 min), cdc15p returned to a hyperphosphorylated form (Figure 5) and was seen as a ring associated with the site of septum synthesis (see Figures 3A and 3C). A similar result was obtained in a synchronous culture of wild-type cells generated by elutriation, when

hypophosphorylated cdc15p was seen just prior to the septation peaks (data not shown). Thus, the hypophosphorylated forms of cdc15p predominate before septation, at times when the actin ring is being formed and cdc15p staining is coincident with F-actin. During septation, when the cdc15p ring contracts, cdc15p returns to the hyperphosphorylated form.

The Abundance of *cdc15* mRNA Increases before Septum Formation

Synchronously dividing cells were generated by arrest–release of a *cdc25-22* mutant. The abundance of *cdc15* mRNA increased shortly after shift to 25°C (30–45 min; Figure 6), before the peak of anaphase (60 min), declining to a low level before septation (90 min). This result was confirmed using a synchronous culture generated by centrifugal elutriation, in which the abundance of *cdc15* mRNA was low in G2 cells and increased significantly just before the peaks of septum formation (data not shown). Analysis of RNA from cells arrested at different stages of the cell cycle showed that the level of *cdc15* mRNA was low in G2-arrested cells (*cdc2-17* and *cdc25-22*) and high in the *nda3-KM311* mutant, which is arrested in mitosis (data not shown). Therefore, the abundance of *cdc15* mRNA varies during exponential growth, peaking after the initiation of mitosis but before septation. At present, we do not know whether this results from periodic transcription of the gene, periodic stabilization of a constitutively expressed unstable mRNA, or a combination of these.

Expression of the *cdc15* Gene in G2-Arrested Cells Promotes Actin Rearrangement to the Center of the Cell

Septum formation is dependent upon the initiation, although not completion, of mitosis (Nurse et al., 1976; Minet et al., 1979). Since formation of the actin ring, increase in abundance of *cdc15* mRNA, and the appearance of hypophosphorylated cdc15p also depend upon initiation of mitosis, we tested whether expression of the *cdc15* gene in a G2-arrested cell could promote F-actin rearrangement. We constructed a strain carrying a single integrated copy of the *cdc15* gene expressed from the *nmt1* promoter and crossed it into *cdc2-17* and *cdc25-22* mutants. Expression of *cdc15* was induced at 25°C, and cell-cycle arrest was imposed by shift to 36°C. Cells in which *cdc15* expression was not induced showed a normal G2 arrest, with F-actin located at the tips of cells (Figure 7A). In contrast, increased expression of *cdc15* caused actin rearrangement in the arrested cells (Figure 7A). In a *cdc25-22* background, 27% of cells had rearranged actin to the middle of the cell, compared with 2% in the uninduced control, while in a *cdc2-17* background, 36% of cells had displaced actin to the center of the cell, compared with 4% in the uninduced control (Figure 7B). No deposition of septum material was seen in the induced cells. Fluorescence-activated cell sorter analysis indicated that both *cdc25-22* and *cdc2-17* arrested with 2C DNA content, whether *cdc15* expression was induced or not (data not shown).

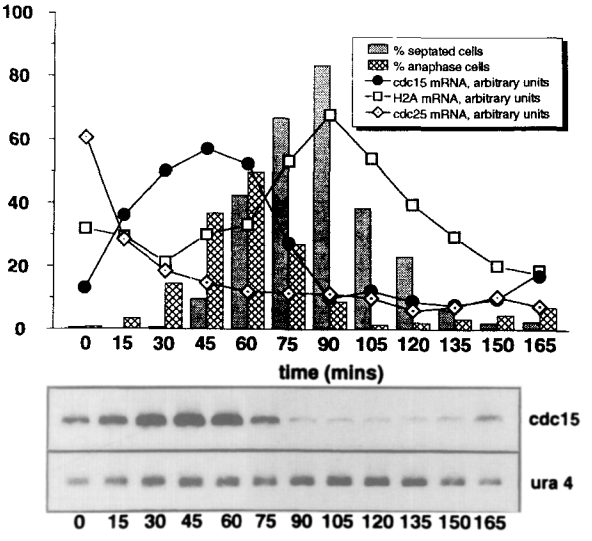

Figure 6. The Abundance of *cdc15* mRNA Increases before Septum Formation

Changes in the steady-state level of the *cdc15* mRNA in a synchronous population of *cdc25-22* cells. *cdc25-22* cells were synchronized, and samples were taken for RNA and microscopic examination. A Northern blot was probed for *cdc25*, *cdc15*, *ura4*, and histone H2A. The blots were quantified using a PhosphorImager. Relative amounts of *cdc15*, *cdc25*, and histone H2A mRNAs compared with *ura4* are shown.

Kinase assays were performed to determine whether F-actin rearrangement was accompanied by activation of $p34^{cdc2}$. No difference in $p34^{cdc2}$ kinase was seen in *cdc25-22*-arrested cells, whether or not *cdc15* expression was induced (Figure 7C), and the level remained much lower than that seen in mitotically arrested cells (*nda3-KM311*; 6 hr arrest). $p34^{cdc2}$ kinase activity was very low in extracts of *cdc2-17*-arrested cells, in which the kinase is inactive at 36°C. This shows that ectopic expression of *cdc15* in a G2-arrested cell can bypass the normal dependency of actin rearrangement upon initiation of mitosis and that activation of $p34^{cdc2}$ kinase is not required for formation of the actin ring.

Strong Expression of Cdc15p Inhibits Formation of the Medial Actin Ring

In the experiment described above, we noted that while some cells had rearranged actin to form a medial ring, others formed an aggregate in the middle of the cell. We therefore examined the effect of prolonged induction of *cdc15* expressed from the *nmt1* promoter in a wild-type background (Figure 7D). Cells expressing increased levels of *cdc15* were not able to form a colony. Staining of these cells showed that although actin rearrangement to the cell center occurred, it formed an aggregate rather than a ring (Figure 7D). Some residual staining was also seen at the tips of the cells. Staining for cdc15p showed that it formed dots at the center of the cell rather than the ring seen in normal cells (Figure 7D). Thus, high level expression of cdc15p blocks septation by interfering with actin ring formation.

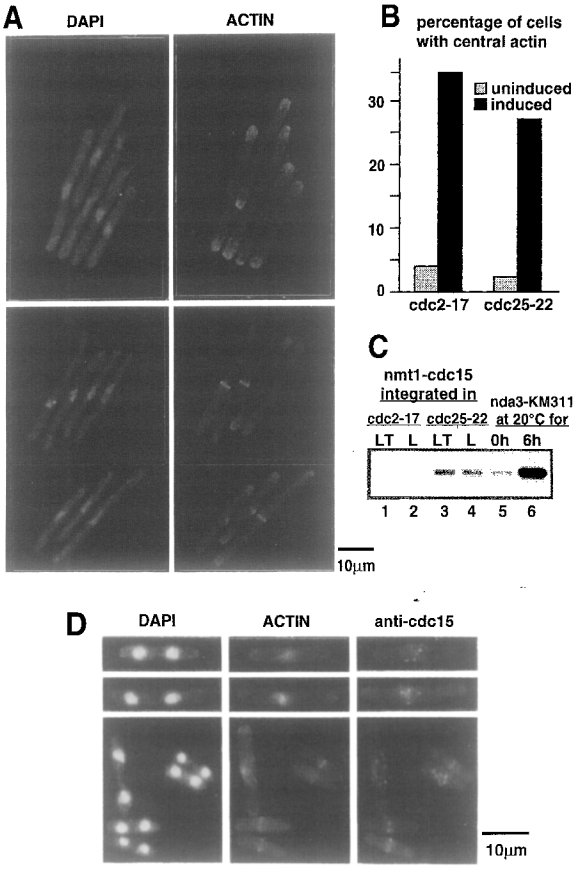

Figure 7. Overproduction of *cdc15* Bypasses the Normal Dependency of Actin Rearrangement upon Entry into Mitosis

(A) Cells were grown to midexponential phase in EMM2 containing leucine and thiamine, washed twice in thiamine-free medium, and half of the cells were resuspended in medium containing thiamine, while the other half were induced to overproduce *cdc15* by incubation in thiamine-free medium. Cells were grown for 12 hr at 25°C, shifted to 36°C for 4 hr, and then fixed with 4% paraformaldehyde and stained with rhodamine-conjugated phalloidin, DAPI, and calcofluor. The percentage of cells with central actin is shown for each strain. Fields of cells stained for DNA or F-actin are shown for the *cdc25-22* mutant. The upper pair of images are uninduced cells; the lower pair are induced cells. Similar results were obtained in a *cdc2-17* background (data not shown).

(B) Graphic representation of the result of expression of cdc15p in G2-arrested cells. Bars indicate the percentage of cells that had rearranged actin to the center of the cell. At least 300 cells were counted for each sample.

(C) Cells were grown as in (A), and protein extracts and histone H1 kinase assays were performed as described in Experimental Procedures. A control sample with mitotic levels of histone H1 kinase activity was generated by incubating the cold-sensitive strain *nda3KM311* at 20°C for 6 hr.

(D) Wild-type cells carrying a single copy of the integrated *cdc15* gene under the control of the *nmt1* promotor were induced for 18 hr at 29°C in EMM2. Cells were fixed and stained for F-actin, cdc15p, and DNA.

Genetic Interactions between *cdc15* and Other Septation Mutants

Mutants in *cdc3*, *cdc4*, *cdc8*, and *cdc12* do not perform a normal actin rearrangement at the onset of mitosis (Marks et al., 1987; Balasubramanian et al., 1992, 1994). Double mutants between these strains and *cdc15-140* were constructed to examine whether there were any additive effects. In contrast with the parent strains, the double mutant *cdc4-8 cdc15-140* was unable to form colonies at either 25°C or 29°C. Shifting cells from the permissive temperature, 19°C, to 29°C resulted in formation of elongated multinucleate cells, which did not form a division septum. The *cdc3-6 cdc15-140* mutant was elongated at both 25°C and 29°C, but could form colonies slowly at both temperatures. No significant differences in behavior were found between *cdc8-110 cdc15-140* or *cdc12-112 cdc15-140* and the respective parent strains (data not shown). These observations suggest that cdc3p, cdc4p, and cdc15p may cooperate to bring about the actin rearrangements required for cytokinesis.

Discussion

Eukaryotic cells must ensure proper temporal and spatial coordination of cytokinesis with mitosis, as division of the cell before chromosome segregation has been completed may result in loss of genetic information, which can in turn lead to cell death or loss of growth control (reviewed by Hartwell, 1992). Study of the fission yeast S. pombe provides a simple system in which to address this problem. Analysis of mutants defective in one of the first steps in cytokinesis, reorganization of the actin cytoskeleton to form the medial actin ring, is of particular interest as they may identify initiators of cytokinesis or the targets of these regulatory mechanisms. We have studied the actin rearrangements in the so-called early septation mutants (Nurse et al., 1976), which are unable to form a division septum, and have found that loss of *cdc15* function impairs actin ring formation.

Phosphorylation has been implicated in regulating events in cytokinesis in higher eukaryotes (reviewed by Satterwhite and Pollard, 1992; Margolis and Andreassen, 1993; Fishkind and Wang, 1995) and also plays a crucial role in the control of cytokinesis and cell polarity in fission yeast, where the activity of kinases and phosphatases is required for both initiation and completion of septum formation and cytokinesis (reviewed by Simanis, 1995). The work presented here strongly suggests that changes in phosphorylation will play an important role in regulating the activity of cdc15p. Initiation of mitosis and actin rearrangement is correlated with appearance of hypophosphorylated cdc15p, suggesting that it is this form of cdc15p that participates in actin ring formation. The role of cdc15p in actin ring formation is not yet clear, but hypophosphorylated cdc15p could be required to stabilize the actin ring, or it may provoke rearrangement of actin to the center of the cell. In the latter case, the interphase form of cdc15p may be maintained in an inactive state by phosphorylation to inhibit actin reorganization from the ends, where it is associated with sites of cell wall deposition and growth. As anaphase is completed and septation is initiated, cdc15p becomes hyperphosphorylated. At this time, while the actin staining changes from a ring to a series of dots on either side of the septum, cdc15p remains visible as a ring, which becomes smaller as synthesis of the septum proceeds. The decrease in size of this ring correlates with

reappearance of the hyperphosphorylated form of cdc15p. Rephosphorylation of cdc15p as the septum is formed may allow the ring to contract or prevent the formation of any more actin rings. Mapping and mutagenesis of the phosphorylation sites on cdc15p, and identification of the kinases and phosphatases that act upon them, will be of considerable interest.

The nature of the ring structure of which cdc15p is a component is unknown at present, but it is tempting to speculate that it is the fission yeast equivalent of the mammalian contractile ring and midbody. Reconstruction of serial electron microscopic sections has shown that as the septum develops it is surrounded by fine fibrils and microfilaments and that there are microtubules crossing the isthmus of the dividing cytoplasm at this point (Kanbe et al., 1989). It will be of interest to determine whether cdc15p is associated with these filaments.

The staining pattern of cdc15p contrasts with those of cdc3p (Balasubramanian et al., 1994) and cdc8p (Balasubramanian et al., 1992). Cdc3p colocalizes with both tip and medial F-actin. In septated cells, it is seen in the medial region as a broad band rather than the ring-like staining we observed with cdc15p (the formaldehyde/glutaraldehyde fixation protocol used for cdc3p gives results for cdc15p staining similar to those presented above; see Figure 3). Cdc8p also colocalizes with actin at all stages of the cell cycle and appears at the cell equator after formation of the actin ring. It is possible that cdc3p and cdc8p are implicated in organization of F-actin at all stages of the cell cycle, while cdc15p may function specifically in formation of the medial ring. The finding that ectopic expression of cdc15p promotes reorganization of actin to the center of the cell is consistent with this view.

cdc4 cdc15 and, to a lesser extent, *cdc3 cdc15,* double mutants have reduced restrictive temperatures compared with the parental strains. *cdc3* encodes fission yeast profilin, while *cdc4* shows homology to myosin regulatory light chain, both of which interact with actin and are required for cytokinesis in yeast and higher eukaryotes (Balasubramanian et al., 1994; McCollum et al., 1995; Karess et al., 1991; Haugwitz et al., 1994). These observations suggest that the products of the *cdc3*, *cdc4*, and *cdc15* genes may cooperate in bringing about the actin rearrangements in cytokinesis. Future experiments will investigate whether cdc15p is an actin-binding protein and whether it interacts with cdc4p, cdc3p, or both.

The N-terminal coiled-coil region and the C-terminal SH3 domain in cdc15p both provide potential for interaction with other proteins. SH3 domains are present in a wide variety of proteins and interact with proline-rich motifs to promote specific protein–protein interactions. SH3 domain–containing proteins have been implicated both in signal transduction pathways and in cell polarization and cytoskeletal organization. A feature of many SH3 domain proteins is that they are associated either permanently or transiently with the plasma membrane (reviewed by Mussachio et al., 1994), as is the case for cdc15p during actin ring formation. This work has demonstrated that cdc15p function is required for proper organization of actin at mitosis. SH3 domain–containing proteins have also been implicated in cytoskeletal organization in S. cerevisiae. Sla1p (Holtzman et al., 1993), Bem1p (Chenevert et al., 1992), and Rvs167p (Bauer et al., 1993) are all required for proper formation of the cortical-actin cytoskeleton. In addition, overexpression of the SH3-containing actin-binding protein Abp1p grossly perturbs the cytoskeleton (Drubin et al., 1988, 1990). The role of the SH3 domain in cdc15p function is unknown at present, but preliminary data indicate that *cdc15* truncations lacking this domain are unable to rescue the *cdc15* deletion mutant. Future experiments will be directed toward identification of the cdc15p SH3 domain ligand.

The role of the periodic accumulation of *cdc15* mRNA is unknown at present. However, the presence of the PEST region in cdc15p raises the possibility that the appearance of the hypophosphorylated form of cdc15p after the onset of mitosis arises from de novo synthesis of the protein from the increased level of *cdc15* mRNA, coupled with destruction of the hyperphosphorylated cdc15p, rather than dephosphorylation of existing cdc15p. Future experiments will investigate this.

Expression of cdc15p in G2-arrested cells promotes actin reorganization to the cell equator, bypassing the usual requirement for initiation of mitosis. This does not depend upon $p34^{cdc2}$ kinase activity. Cells that formed an actin ring in G2 after overproduction of cdc15p did not form a division septum, suggesting that additional signals or proteins that are required for septum formation are absent in G2-arrested cells or that the actin ring that was made was aberrant and the septum could not be formed. High level expression of *cdc15* blocked formation of the division septum and caused cell-cycle arrest. F-actin in these cells was reorganized to the center of the cell, but formed a disorganized aggregate, and cdc15p was seen as dots rather than a ring at the middle of the cell. An excess of cdc15p may interfere with normal actin ring assembly if formation of the actin ring requires the correct ratio of cdc15p to actin (or some other protein). Overproduction of cdc3p (profilin) or Drosophila melanogaster profilin in S. pombe also causes cell-cycle arrest and shape defects. However, no discrete F-actin staining was detected in cells overproducing profilin, suggesting a general depolymerization rather than relocalization of F-actin (Balasubramanian et al., 1994; Edwards et al., 1994).

In higher eukaryotes, it has been demonstrated that the position of the mitotic spindle is important for determining the site of cell cleavage and also that multiple regions in the cortex are capable of furrow formation. Removal of the mitotic spindle during anaphase or later does not prevent cytokinesis, implying that the position of the cleavage furrow had been determined by anaphase. In sea urchin eggs, it has been demonstrated that the asters of the spindle dictate where the cleavage furrow will form (reviewed by Rappaport, 1986; Satterwhite and Pollard, 1992; Strome, 1993). In fission yeast, little is known about the signals that define where the actin ring should form so that the septum is synthesized in the middle of the cell. It is noteworthy that the actin ring formation promoted by ectopic expression of cdc15p in G2 cells occurs at the cell equator, suggesting that the location where the actin ring

will form has already been defined in a G2 cell. At present, we do not know whether assembly of cdc15p at the middle of the cell depends upon the presence of actin. The nature of the template that defines where cdc15p and actin will assemble will be of considerable interest.

Some of the proteins identified by analysis of S. pombe cytokinesis mutants (reviewed by Fankhauser and Simanis, 1994a) have been conserved through evolution, both structurally and functionally. For example, expression of a murine tropomyosin gene in fission yeast rescues an S. pombe *cdc8* null mutation (Balasubramanian et al., 1992), and mutation of the Drosophila *spaghetti-squash* gene, which encodes nonmuscle myosin light chain (Karess et al., 1991), or a deficiency of profilin in Dictyostelium amoebae both cause defects in cytokinesis (Haugwitz et al., 1994), emphasizing the universal importance of these proteins for cell division. The product of the Drosophila *peanut* gene, which is required for cytokinesis, is similar to S. cerevisiae *Cdc3p*, *Cdc10p*, *Cdc11p*, and *Cdc12p*, which are components of the 10 nm filaments found at the bud neck of budding yeast (Neufeld and Rubin, 1994). Homologs of these genes have also been identified in S. pombe, mouse, and human cells (Nakatsuru et al., 1994; Neufeld and Rubin, 1994), suggesting they may be important for cytokinesis in all eukaryotes. The identification of proteins that have structural similarity to cdc15p suggests that its function may also have been conserved through evolution. At present, no specific function has been ascribed to the S. cerevisiae, mouse, or tapeworm proteins. It will be of interest to determine whether they have a *cdc15*-like function and whether homologs of *cdc15* exist in other organisms.

Experimental Procedures

S. pombe Methods

Standard techniques were used for growth, manipulation, and synchronization of fission yeast (Moreno et al., 1991). Cells were grown in yeast extract (YE) or EMM2 minimal medium, supplemented as required. Cells were grown in EMM2 medium for all synchronization experiments. Selection synchrony was performed using a Beckman JS5.0 elutriation system. Induction synchrony was performed by arrest–release of a *cdc25-22* mutant. In brief, cells were grown to midexponential phase at 25°C and then shifted to 36°C for 4 hr. The culture was cooled rapidly to 25°C, and samples were removed for analysis of RNA or protein. Construction and induction of a single-copy *nmt1* promoter–driven integrant of the *cdc15* cDNA at the *leu1* locus using the pINT5 vector was performed as described previously (Fankhauser and Simanis, 1994b). Cell number was determined with a Coulter counter and percentage of septated cells by dark-field microscopy or staining with calcofluor.

Cloning of the *cdc15* Gene

The *cdc15* gene was cloned by rescue of the heat sensitivity of the strain *cdc15-140 ura4D18 h*$^-$ after transformation with a wild-type gene library (Barbet et al., 1992). Standard techniques for DNA manipulation were used throughout (Sambrook et al., 1989). Integrative mapping, to check that the *cdc15* gene rather than an extragenic suppressor had been cloned, was performed as follows. A stable integrant of the *cdc15* plasmid, which carries the *ura4*$^+$ gene, was obtained in a *cdc15-140 ura4D18* strain. This was crossed to a *cdc15*$^+$ *ura4D18* strain. No *cdc*$^-$ *Ura*$^+$ progeny were observed in dissection of 59 tetrads or 524 colonies in free spore analysis, demonstrating very tight linkage between the integrated *ura4*$^+$ gene and the *cdc15* locus. In addition, crosses between the integrant and an *ade3-58 ura4D18* strain gave 23 PD, 6 TT, and 0 NPD (scoring linkage of the integrated *ura4* marker to *ade3*), consistent with the previously described linkage between *cdc15* and *ade3* (Kohli et al., 1977). Physical mapping of the gene against an ordered cosmid library (Hoheisel et al., 1993) showed hybridization to cosmid 20G8 from chromosome I.

cdc15 Deletion

To disrupt *cdc15*, we replaced a HpaI–BglII fragment (Figure 2A) with the *ura4* gene. A fragment produced by digestion with PvuII and XbaI was used to transform a diploid constructed by mating *ade6M210* and *ade6M216* strains, which complement in *trans*. Correct integration was verified by Southern blotting.

Production of Antisera, Protein Extracts, and Immunoprecipitation

A BalI–SalI fragment (corresponding to amino acids 80–320 of cdc15p) was ligated into EcoR1-digested pGEX-3X after both had been blunt ended with Klenow fragment. The fusion protein was produced in DH5α, solubilized from inclusion bodies, purified by preparative SDS–PAGE (Fankhauser and Simanis, 1993), emulsified with incomplete Freund's adjuvant, and approximately 200 μg was injected monthly into New Zealand white rabbits. Standard procedures for antibody production and assay were used throughout (Harlow and Lane, 1988). For Western blotting, bound antibodies were detected using secondary antibodies coupled to peroxidase and revealed with ECL (Amersham) or with alkaline phosphatase–coupled antibodies. Soluble protein extracts and immunoprecipitations were performed as previously described (Fankhauser and Simanis, 1994b), except that anti-cdc15p antiserum was used. p34^{cdc2} kinase assays were performed as described in Moreno et al. (1989).

CIP Treatment

Soluble protein extract (1 mg) from *cdc25-22*-arrested cells was immunoprecipitated as described above and then split in two. One half was washed twice with calf intestine phosphatase (CIP) buffer (100 mM Tris–HCl [pH 9.6], 2 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1% aprotinin, 1 mM PMSF, 20 μg/ml leupeptin), resuspended in 10 μl of CIP buffer containing 10 U of CIP (Boehringer Mannheim), and incubated for 15 min at 37°C. The reaction was stopped by the addition of 15 μl of 2× SDS gel–loading buffer and boiling for 3 min. For the mock treatment, the immunoprecipitate was washed twice with CIP buffer plus 15 mM EGTA, 15 mM paranitrophenylphosphate, 5 mM NaF, and 60 mM β-glycerolphosphate and resuspended in 10 μl of the same buffer containing 10 U of CIP, which was heat treated for 10 min at 65°C prior to addition to the immunoprecipitate (adapted from Cairns et al., 1992).

Microscopy

Staining for F-actin was performed as previously described (Marks and Hyams, 1985). For *cdc15* immunolocalization, cells were fixed by addition of paraformaldehyde exactly as for F-actin staining. In brief, cells were fixed by addition of 1/7 volume of 30% (w/v) formaldehyde in 100 mM PIPES (pH 6.9), 1 mM EGTA, 1 mM $MgSO_4$ (PEM). After 60 min, cells were washed three times with PEM, once with PEM plus 1% Triton X-100, and then three times with PEM. Cells were resuspended in PEM plus 1.2 M Sorbitol (PEMS) containing 2.5 mg/ml zymolyase 20T (Seikagaku Corporation) and incubated for 70 min at 37°C. They were washed once in PEMS plus 1% Triton X-100 and three times in PEM, resuspended in PEM plus 1% BSA (Sigma A-7030), 100 mM lysine hydrochloride, 0.1% sodium azide (PEMBAL), and gently shaken for 30 min. Cells (5×10^6) were then resuspended in 80 μl of PEMBAL containing 2 μl of affinity-purified *cdc15* antibody and left on a rotating wheel overnight. After three 10 min washes in PEMBAL, cells were resuspended in FITC-conjugated secondary antibodies diluted 1/50 in PEMBAL (Nordic Immunology) and incubated 4 hr at 37°C. They were then washed once in PEM, once in PBS (pH 8.0), once in PBS (pH 8.0) plus 1 μg/ml DAPI (Sigma), and finally resuspended in 50 μl of PBS (pH 8.0) plus 0.1% sodium azide and 2 μg/ml rhodamine-conjugated phalloidin (Sigma) and 20 μg/ml calcofluor (fluorescent brightener number 28; Sigma). They were then mounted in 50% glycerol containing 1 mg/ml paraphenylene diamine and observed with a Zeiss Axiophot microscope. For competition experiments, 80 μl of affinity-purified serum (1/40) was preincubated for 60 min with 3 μg of GST or 3 μg of GST–cdc15p fusion protein prior to addition to the cells. Similar staining patterns were obtained after

fixation with 3% paraformaldehyde plus 0.2% glutaraldehyde, although the intensity of the signal was diminished (data not shown).

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